MT1-MMP mediates MUC1 shedding independent of TACE/ADAM17

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MUC1, a transmembrane mucin, plays a critical role in embryo implantation, protection of mucosal epithelia from microbial and enzymic attack and various aspects of tumour progression. In some species, a decrease in uterine epithelial MUC1 protein and mRNA expression accompanies embryo implantation. In other species, such as rabbits and humans, MUC1 appears to be locally removed at blastocyst attachment sites, suggesting the action of a protease. We previously demonstrated that MUC1 is proteolytically released from the surface of a human uterine epithelial cell line, HES, and identified TACE/ADAM17 (where TACE stands for tumour necrosis factor- α converting enzyme and ADAM for A Disintegrin And Metalloprotease-like) as a constitutive and PMA-stimulated MUC1 sheddase [Thathiah, Blobel and Carson (2003) J. Biol. Chem. **274**, 3386–3394]. Further characterization of the proteolytic activity(ies) mediating MUC1 release indicates that MUC1 shedding is also accelerated by the tyrosine phosphatase inhibitor pervanadate. Pervanadate, but not PMA, stimulates MUC1 shedding in TACE-deficient cells, indicating

activation of a metalloproteolytic activity(ies) distinct from TACE. Pervanadate-stimulated MUC1 release is inhibited by the TIMP-2 (tissue inhibitor of metalloprotease-2) and TIMP-3, but is unaffected by TIMP-1, consistent with the MT-MMPs (membrane-type matrix metalloproteases). Pervanadate stimulation of MUC1 shedding is absent from MUC1-transfected MT1-MMP-deficient fibroblasts, but is restored after MUC1 and MT1-MMP co-transfection. Furthermore, overexpression of MT1-MMP in HES cells enhances pervanadate-stimulated MUC1 release, and MT1-MMP co-localizes with MUC1 *in vivo* at the apical surface of receptive-phase human uterine epithelia. Taken together, these studies characterize a MUC1 sheddase activity in addition to TACE and identify MT1-MMP as a pervanadate-stimulated MUC1 sheddase.

Key words: endometrium, implantation, metalloprotease, MT1-MMP, MUC1, TACE/ADAM17.

INTRODUCTION

Ectodomain proteolysis, or ectodomain shedding, has a significant impact on the biological activity of several integral membrane proteins including cytokines/growth factors and their receptors, cell-adhesion molecules and ectoenzymes. More specifically, ectodomain shedding affects the nature of cell signalling events by regulating the availability of active, soluble forms of ligands and/or receptors and by altering signalling cascades from autocrine or juxtacrine to paracrine or endocrine events (reviewed in [1]). Removal of extracellular domain has been implicated in the process of cell-fate determination, cell migration and skeletal development (reviewed in [2]), and is associated with the progression of several disease processes, including Alzheimer's disease [3], osteoarthritis [4] and cancer progression [5].

Despite differences in structure, membrane topology, tissue distribution and cleavage-site sequence, protein ectodomain shedding is predominantly inhibited by metalloprotease inhibitors [6]. Moreover, correlative studies have shown that members of the ADAM (A Disintegrin And Metalloprotease-like) family of metalloproteases mediate proteolytic processing of several membrane-anchored proteins, including TNF α (tumour necrosis factor- α), TNFR1 (TNF receptor-I) and TNFR2, transforming growth factor- α , HER4 (human epidermal growth factor receptor-4), L-selectin, amyloid precursor protein and cellular prion protein (reviewed in [2,7]). These studies demonstrate an essential role for ADAMs in protein ectodomain shedding; however, MMPs (matrix metalloproteases) also have a functionally relevant role

in proteolytic release of membrane-anchored proteins. In this regard, stromelysin-1 (MMP-3) mediates release of soluble, active heparin-binding epidermal growth factor [8]. Matrilysin (MMP-7) processes TNF α from macrophages [9]. MMP-17 (MT4-MMP, a membrane-type 4 MMP) also mediates release of TNF α [10] and MMP-14 (MT1-MMP) has been implicated in the proteolytic processing of TRANCE (TNF-related activation-induced cytokine) [11], CD44 [12] and tissue transglutaminase [13]. These studies indicate that a single protein can be processed by different proteases and, in contrast, that one protease can process multiple membrane proteins.

MUC1, a transmembrane mucin glycoprotein, is an extremely effective inhibitor of embryo attachment to uterine epithelia [14]. Reduced MUC1 mRNA expression throughout the luminal uterine epithelium accompanies uterine receptivity to blastocyst attachment in most of the species examined (reviewed in [15]). In contrast, Muc1 expression is increased during the peri-implantation period in rabbits; however, careful examination of rabbit implantation sites *in vivo* and *in vitro* has revealed that Muc1 is lost solely at the site of embryo–uterine apposition [16]. Interestingly, increased expression of ADAM9 accompanies Muc1 loss at implantation sites in rabbits [17], implicating ADAM9 in the implantation process in this species. Uterine MUC1 in humans also appears to be increased during the receptive phase [18]. Although implantation sites have not been studied, *in vitro* implantation models indicate that MUC1 also is lost at the site of embryo attachment in humans [19], suggesting that factors expressed on the blastocyst surface or released with limited diffusibility trigger

Abbreviations used: ADAM, A Disintegrin And Metalloprotease-like; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; D-PBS, Dulbecco's phosphate-balanced saline; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; HER, human epidermal growth factor receptor; MMP, matrix metalloprotease; MT-MMP, membrane-type MMP; PKC, protein kinase C; RT, reverse transcriptase; TNF, tumour necrosis factor; TACE, TNF α -converting enzyme; TAPI, TNF α protease inhibitor; TNFR, TNF receptor; TRANCE, TNF-related activation-induced cytokine; TIMP-1, tissue inhibitor of metalloprotease-1.

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MUC1 loss. Recently, we demonstrated that MUC1 is proteolytically released from the surface of a human uterine epithelial cell line, HES, and implicated TACE (TNF α -converting enzyme) as a constitutive and PMA-stimulated MUC1 sheddase [20]. PMA is an activator of PKC (protein kinase C) and, therefore, these findings also implicated PKC as an upstream activator of MUC1 proteolytic release. The aim of the present study was to determine the possible involvement of additional proteolytic activities in MUC1 ectodomain shedding. Specifically, MUC1 sheddase activities were assessed in response to stimuli with the potential to induce ectodomain shedding independent of PKC activation. Utilization of the protein tyrosine phosphatase inhibitor pervanadate to sustain protein phosphorylation-dependent events indicates that pervanadate stimulates MUC1 release from HES uterine epithelial cells and from TACE-deficient cells, but not MT1-MMP-deficient murine embryonic fibroblasts, transfected with MUC1 cDNA. Furthermore, overexpression of MT1-MMP enhances MUC1 release from HES cells, and MT1-MMP and MUC1 are co-localized *in vivo* at the apical aspect of human uterine epithelia from the receptive-phase endometrium. Our results demonstrate that, in addition to TACE, MT1-MMP can mediate MUC1 ectodomain release, suggesting that multiple pathways can impact MUC1 stability at the cell surface.

MATERIALS AND METHODS

Materials

Sodium vanadate and the furin inhibitor decanoyl-RVKR-CMK were purchased from Calbiochem. The recombinant catalytic domain of MT1-MMP, GM6001 (Ilomastat) and rabbit anti-MT1-MMP polyclonal antibody, specific for the hinge region, were purchased from Chemicon (Temecula, CA, U.S.A.). Leupeptin, pepstatin A, E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] and H₂O₂ were obtained from Sigma. TIMP-1 (tissue inhibitor of metalloprotease-1), TIMP-2, TIMP-3 and recombinant human TACE were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Protein G-Sepharose, Texas Red-conjugated sheep anti-mouse IgG and FITC-conjugated donkey anti-rabbit IgG were purchased from Amersham Biosciences. DAPI (4',6-diamidino-2-phenylindole dihydrochloride) was purchased from Molecular Probes. Affinity-purified mouse IgG and rabbit IgG were obtained from Zymed (San Francisco, CA, U.S.A.). A mouse monoclonal antibody specific for a tandem repeat epitope in the extracellular domain of MUC1, 214D4, was kindly provided by Dr J. Hilken (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The metalloprotease inhibitor, TAPI (TNF α protease inhibitor), was kindly provided by Dr J. Doedens and Dr R. Black (Amgen, Seattle, WA, U.S.A.).

cDNA constructs

The TNF α -AP cDNA was generously provided by Dr C. P. Blobel (Sloan-Kettering Institute, New York, NY, U.S.A.). The MT1-MMP cDNA was kindly provided by Dr K. Hotary and Dr S. J. Weiss (University of Michigan, Ann Arbor, MI, U.S.A.). Full-length human MUC1 cDNA, a gift from Dr S. Gendler (Mayo Clinic, Scottsdale, AZ, U.S.A.), was ligated into the expression vector pcDNA3.1 (Invitrogen) for use in transfection experiments.

RT (reverse transcriptase)-PCR

Total RNA was extracted from uterine epithelial HES cells using the RNeasy kit according to the manufacturer's instructions

(Qiagen, Chatsworth, CA, U.S.A.). Total RNA was extracted from frozen human endometrial tissue samples using the TRIzol[®] reagent according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD, U.S.A.). Quantification and estimation of purity were performed by measuring the absorbance of each RNA sample at wavelengths of 260 and 280 nm, and integrity was determined by visual inspection of RNA fractionated by agarose gel electrophoresis. Reverse transcription was performed using the Advantage RT-for-PCR kit according to the manufacturer's instructions (Clontech Laboratories, Palo Alto, CA, U.S.A.). PCR was performed using the HotStarTaq Master Mix kit according to the manufacturer's instructions (Qiagen).

Cell culture and MUC1 shedding assay

The human uterine epithelial cell line HES was kindly provided by Dr D. Kniss (Ohio State University, Columbus, OH, U.S.A.). HES cell culture and MUC1 shedding assays were performed as described previously [20]. Pervanadate was prepared immediately preceding use by combining 100 mM VO₄ and 100 mM H₂O₂. At the time of treatment, culture medium was replaced with fresh serum-free medium in the presence or absence of 100 μ M pervanadate and one of the following protease inhibitors: leupeptin (10 μ M), pepstatin A (10 μ M), E-64 (10 μ M), TAPI (100 μ M), GM6001 (25 μ M), decanoyl-RVKR (25 μ M), TIMP-1 (20 μ g/ml), TIMP-2 (20 μ g/ml), TIMP-3 (20 μ g/ml) or the appropriate vehicle control. After 1 h incubation, the cells were examined by phase microscopy for survival and morphology, and cell lysates and culture supernatants were collected for Western-blot analysis. In all cases, cell viability exceeded 95 % by the Trypan Blue exclusion assay.

HES cells were electroporated using a Bio-Rad GenePulser II with a capacitance extender in 0.4 cm cuvettes at 230 V and 500 μ F. Three million cells were used for each electroporation assay. Cells were transfected with 12 μ g of the MT1-MMP expression plasmid or with empty vector cDNA. After electroporation, cells were transferred to Matrigel-coated 6-well plates and allowed to recover for 24 h in complete growth medium at 37 °C. At the time of treatment, culture medium was replaced with fresh serum-free medium in the presence or the absence of 100 μ M pervanadate or the appropriate vehicle control. After a 1 h incubation, the cells were examined by phase microscopy for survival and morphology, and cell lysates and culture supernatants were collected for Western-blot analysis.

Wild-type EC-4 and TACE-deficient EC-2 murine fibroblasts [21] were kindly provided by Dr J. Doedens and Dr R. Black and were cultured in Dulbecco's modified Eagle's medium/F12 (Life Technologies) supplemented with 1 % (v/v) fetal bovine serum (Life Technologies), 100 units/ml penicillin and 100 μ g/ml streptomycin. Wild-type and MT1-MMP-deficient murine embryonic fibroblasts were kindly provided by Dr K. Hotary and Dr S. J. Weiss and were cultured in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10 % (v/v) foetal calf serum (Life Technologies), 100 units/ml penicillin and 100 μ g/ml streptomycin. Cells were electroporated using a Bio-Rad GenePulser II and allowed to recover overnight as described previously [20]. At the time of treatment, culture medium was replaced with fresh serum-free medium in the presence or the absence of 100 μ M pervanadate and one of the following protease inhibitors: TAPI (50 μ M), decanoyl-RVKR (25 μ M), TIMP-1 (100 nM), TIMP-2 (100 nM), TIMP-3 (100 nM) or the appropriate vehicle control. After a 1 h incubation, the cells were examined by phase microscopy for survival and morphology, and cell lysates and culture supernatants were harvested for Western-blot analysis.

Sample preparation, SDS/PAGE and detection of MUC1 protein

Sample preparation, protein determination, SDS/PAGE and Western-blot analysis of MUC1 proteins were performed as described previously [20]. Statistical analyses were performed using one-way ANOVA and the Tukey–Kramer multiple comparisons test (GraphPad InStat program).

TNF α shedding assay

HES cells were electroporated as described above. Briefly, cells were transfected with 12 μ g of the TNF α -AP expression plasmid or with empty vector. After electroporation, cells were transferred to Matrigel-coated 24-well plates and allowed to recover for 24 h in complete growth medium at 37 °C and treated as described above. After a 1 h incubation, photometric quantification of released TNF α -AP in culture supernatants was performed as described previously [22].

Immunofluorescence

Endometrial specimens were obtained during routine fertility evaluations of healthy women with a regular menstrual cycle between the ages of 18 and 45 years. Samples were dated histologically by an experienced gynaecological pathologist and by serum hormone profiles. Samples were frozen immediately in liquid nitrogen and embedded in OCT cryoprotectant (Baxter, Deerfield, IL, U.S.A.) for cryosectioning. The use of human subjects was approved by the Institutional Review Board of the University of Delaware.

Frozen mid-luteal human endometrial sections (8 μ m) were fixed for 10 min at room temperature (21–22 °C) in methanol and rehydrated in D-PBS (Dulbecco's phosphate-balanced saline) for 10 min with one change of buffer. Sections and cells were incubated at 37 °C for 1 h with anti-MT1-MMP polyclonal antibody diluted to 5 or 10 ng/ μ l in D-PBS. Non-immune rabbit IgG was used as a negative control. Samples were rinsed three times for 5 min in D-PBS at room temperature and incubated at 37 °C for 40 min with FITC-conjugated donkey anti-rabbit IgG diluted 1:10 in D-PBS. After three additional 5 min rinses in D-PBS, samples were incubated for 1 h at 37 °C with anti-MUC1 monoclonal antibody, 214D4, diluted 1:1 in D-PBS or with non-immune mouse IgG. Sections and cells were rinsed three times for 5 min in D-PBS at room temperature and incubated at 37 °C for 40 min with Texas Red-conjugated sheep anti-mouse IgG diluted 1:10 in D-PBS and DAPI. All samples were mounted in glycerol/D-PBS containing 0.01 % (w/v) *p*-phenylenediamine to prevent fading and photographed with a Zeiss LSM 510 Multi-photon Confocal microscope.

In vitro cleavage assay

Synthetic peptides corresponding to 12 amino acids surrounding potential membrane-proximal cleavage sites of MUC1 were prepared commercially (SynPep, Dublin, CA, U.S.A.) at a final concentration of 100 μ M and were incubated in 50 mM Tris/HCl (pH 7.5), 50 mM NaCl, 10 mM CaCl₂ and 0.005 % (v/v) Brij 35 with the recombinant catalytic domain of MT1-MMP (1.5 μ g) in a total volume of 50 μ l in the presence or absence of 5 mM (1,10)-phenanthroline for 6 h at 37 °C. The fluorogenic peptide substrate I (R&D Systems) was used as a positive control for MT1-MMP activity. Similarly, the MUC1 synthetic peptides were incubated in 25 mM Tris (pH 7.5), 2.5 μ M ZnCl₂ and 0.005 % Brij 35 with recombinant TACE (1 μ g) in a total volume of 50 μ l in the presence or absence of 5 mM (1,10)-phenanthroline for 6 h at 37 °C. The TACE substrate II (Calbiochem) was used as a

positive control for TACE activity. Reactions were quenched by the addition of 1 % (w/v) trifluoroacetic acid. The resulting cleavage products were analysed by matrix-assisted laser-desorption ionization–time-of-flight MS using a Bruker Reflex III mass spectrometer (University of North Carolina, Chapel Hill, NC, U.S.A.).

RESULTS

Pervanadate stimulates release of TNF α and MUC1 from HES cells

Pervanadate, a tyrosine phosphatase inhibitor, accelerates ectodomain shedding of various proteins [23,24], including TNF α [11]. To determine whether pervanadate stimulates shedding of MUC1, we initially analysed the effect of pervanadate on the shedding of one of the most intensely studied substrates of TACE, TNF α , in comparison with MUC1. In agreement with previously published observations [11], TNF α shedding is enhanced approx. 3- to 4-fold after pervanadate treatment (Figure 1A). Treatment with vanadate or H₂O₂ alone was ineffective in stimulating TNF α ectodomain release above constitutive levels, indicating the specificity of the pervanadate stimulation. Pervanadate also stimulated shedding of MUC1 comparably (Figures 1B and 1C).

A previous study indicated that ectodomain removal of MUC1 was sensitive to hydroxamate-based metalloprotease inhibitors [20]. To determine whether pervanadate-induced shedding of MUC1 in HES cells is due to metalloproteolytic activation, we examined the effect of two structurally distinct metalloprotease inhibitors on MUC1 shedding. Pervanadate-induced release of MUC1 was inhibited significantly (> 80%) by the hydroxamate-based metalloprotease inhibitors TAPI and GM6001 (Figures 2A and 2B). TAPI also completely inhibited constitutive release of MUC1 in contrast with GM6001, which inhibited MUC1 constitutive release by 60% (Figures 2A and 2B).

To characterize further the pervanadate-stimulated MUC1 sheddase activity, three endogenous metalloprotease inhibitors, TIMP-1, -2, and -3, were examined for their ability to inhibit MUC1 release. Utilizing TIMP concentrations that inhibit metalloprotease activity [25,26] and ectodomain shedding of HER2 [24] and TRANCE [11], TIMP-1 and TIMP-2 failed to inhibit constitutive and pervanadate-stimulated MUC1 release from HES cells (Figures 3A and 3B). In fact, TIMP-2 enhanced MUC1 shedding, an action independent of MMP-2 activation (A. Thathiah and D. D. Carson, unpublished work). Finally, TIMP-3 effectively inhibited pervanadate-induced shedding of MUC1 (Figure 3C). Thus the TIMP inhibition profile was inconsistent with the involvement of the currently known soluble MMPs, which are sensitive to TIMP-1, as constitutive or stimulated MUC1 sheddases in HES cells.

Prodomain removal during biosynthesis is required for proteolytic activity of MMP-11 [27], MMP-28 [28], MT-MMPs [29] and catalytically active ADAMs, [3,30], and appears to be mediated by a furin-type proprotein convertase in the *trans*-Golgi network. Consequently, to determine whether the pervanadate-stimulated sheddase(s) of MUC1 requires processing by furin for activity, a furin inhibitor, decanoyl-RVKR [31], was tested. In contrast with PMA-stimulated MUC1 release [20], decanoyl-RVKR inhibited pervanadate-stimulated MUC1 release completely, but did not affect constitutive release (Figure 4).

TACE is not required for pervanadate-stimulated MUC1 shedding

The above results suggested that different proteolytic activities are responsible for pervanadate- and PMA-stimulated ectodomain release of MUC1 in HES cells. The protease inhibition profiles

Figure 1 Pervanadate stimulates release of MUC1 and TNF α from HES uterine epithelial cells

(A) HES cells, untransfected or transiently transfected with cDNA encoding TNF α -AP, were treated with pervanadate (PV) or controls for the specificity of PV-induced TNF α release, VO₄ and H₂O₂ for 1 h at 37 °C. TNF α -AP recovered from the culture supernatants was then analysed by photometric assay as described in the Materials and methods section. Results are expressed as means $\pm$ S.D. for three independent samples. *** P < 0.001 relative to vehicle control. (B) HES cells were treated with PV or controls for the specificity of PV-induced MUC1 release, VO₄ and H₂O₂ for 1 h at 37 °C. MUC1 expression in cell lysates (lower panel) and culture supernatants (upper panel) was examined by Western-blot analysis using a monoclonal antibody directed against a tandem repeat epitope in the ectodomain of MUC1, 214D4. As a result of differential glycosylation and allelic polymorphism, 214D4 recognizes at least two forms of MUC1. (C) Shed MUC1 was quantified by densitometric analysis and is expressed as a percentage of MUC1 released by cells treated with vehicle alone. Results are expressed as means $\pm$ S.D. for at least three independent samples. * P < 0.05 relative to the corresponding vehicle control. Experiments were performed in triplicate and repeated at least twice with similar results.

implicate members of the MT-MMP and/or ADAM family of metalloproteases as pervanadate-stimulated candidate MUC1 sheddases. In this regard, the examination of shedding events in cells derived from mice genetically deficient for specific ADAM proteases has permitted the identification of potential protease-mediated shedding events [11,21,32,33]. Utilizing embryonic fibroblasts derived from wild-type and *tace* ^{Δ Zn/ Δ Zn} mice [33], we previously demonstrated that, in contrast with wild-type EC-4 cells, TACE-deficient EC-2 cells do not shed MUC1 constitutively or in response to the PMA, implicating TACE as a constitutive and PMA-inducible MUC1 sheddase [20]. To deter-

Figure 2 Pervanadate-stimulated release of MUC1 is sensitive to the synthetic metalloprotease inhibitors TAPI and GM6001

Determination of MUC1 shedding was performed as described in Figure 1. After pretreatment with TAPI or GM6001 for 1 h at 37 °C, HES cells were treated with pervanadate or vehicle in the presence or absence of TAPI (A) or GM6001 (B) for 1 h at 37 °C. MUC1 recovered from the culture supernatants was examined by Western-blot analysis. Results are expressed as means $\pm$ S.D. for three independent samples. ** P < 0.01; *** P < 0.001 relative to vehicle control or pervanadate stimulation. Experiments were performed in triplicate and repeated at least twice with similar results.

mine whether TACE is also a pervanadate-stimulated MUC1 sheddase, MUC1 shedding was examined after electroporation of these cells with MUC1 cDNA or empty vector. Transfected wild-type EC-4 cells behaved similarly to HES cells with respect to constitutive and pervanadate-induced release of MUC1 (Figure 5A). In agreement with our previous findings, TACE-deficient EC-2 cells did not shed MUC1 constitutively [20]. However, TACE-deficient EC-2 cells did shed MUC1 after pervanadate stimulation, indicating a pervanadate-sensitive proteolytic activity distinct from TACE (Figure 5B). Furthermore, the protease(s) was sensitive to inhibition by the peptide hydroxamate metalloprotease inhibitor TAPI (Figures 6A and 6B) and the furin inhibitor decanoyl-RVKR (Figures 6C and 6D), suggesting that the activity is that of a zinc-dependent metalloprotease.

To characterize further the pervanadate-stimulated proteolytic activity(ies) in the wild-type EC-4 and TACE-deficient EC-2 cells, the endogenous metalloprotease inhibitors TIMP-1, -2, and -3 were also evaluated for their ability to inhibit MUC1 ectodomain release. Similar to the HES cells, TIMP-1 failed to inhibit constitutive and pervanadate-induced MUC1 shedding from the wild-type EC-4 and TACE-deficient EC-2 cells (Figures 7A and 7B). Unlike the HES cells, TIMP-2 did not stimulate MUC1 release in this system; however, TIMP-2 as well as TIMP-3 effectively inhibited pervanadate-stimulated release from both cell lines (Figures 7C–7F). Collectively, these results were consistent with the involvement of an MT-MMP(s) in pervanadate-stimulated MUC1 shedding from wild-type EC-4 and TACE-deficient EC-2 cells.

HES cells express MT-MMPs and overexpression of MT1-MMP enhances pervanadate-stimulated MUC1 shedding

MT1- [25], MT2- [34] and MT5- [35] MMP are efficiently inhibited by TIMP-2. MT1- [25] and MT2- [34] MMP are also inhibited by TIMP-3. Together with the above results, these findings implicate an MT-MMP as a pervanadate-stimulated MUC1 sheddase in wild-type EC-4 and TACE-deficient EC-2 cells. To address the potential role of an MT-MMP in pervanadate-stimulated MUC1 shedding in HES cells, we obtained an expression profile of MT-MMPs in HES uterine epithelial cells and in the receptive-phase human endometrium by RT-PCR. The PCR profile demonstrated that MT1-, MT2-, and MT5-MMP transcripts are detectable in both HES cells and in the receptive-phase human endometrium (Table 1). These results, together with the finding that expression of MT1-MMP appears

Figure 3 Pervanadate-stimulated release of MUC1 is differentially sensitive to TIMP-1, -2 and -3

After pretreatment with TIMP-1, -2 or -3 for 1 h at 37 °C, HES cells were treated with pervanadate or vehicle in the presence or absence of TIMP-1 (A), TIMP-2 (B) or TIMP-3 (C) for 1 h at 37 °C. MUC1 recovered from the culture supernatants was examined by Western-blot analysis. Results are expressed as means $\pm$ S.D. for three independent samples. * P < 0.05; *** P < 0.001 relative to vehicle control or pervanadate stimulation. Experiments were performed in triplicate and repeated at least twice with similar results.

to increase in the human endometrium during the receptive phase [36], prompted us to assess the putative role of MT1-MMP as a constitutive and stimulated MUC1 sheddase in the HES uterine epithelial cell line. Overexpression of MT1-MMP in HES cells led to an increase in pervanadate-stimulated MUC1 shedding relative to the vector control transfectants, and modestly, although not significantly, enhanced constitutive MUC1 release (Figure 8).

MUC1 is not shed by MT1-MMP null cells after pervanadate stimulation

Wild-type and MT1-MMP-deficient murine embryonic fibroblasts were utilized to assess the importance of MT1-MMP in MUC1 ectodomain release. Neither the wild-type nor the MT1-MMP null cells express MUC1 endogenously. Therefore MUC1

Figure 4 Pervanadate-stimulated release of MUC1 is sensitive to the furin inhibitor decanoyl-RVGR

After pretreatment with decanoyl-RVGR for 1 h at 37 °C, HES cells were treated with pervanadate or vehicle in the presence or absence of decanoyl-RVGR for 1 h at 37 °C. MUC1 recovered from the culture supernatants was examined by Western-blot analysis. Results are expressed as means $\pm$ S.D. for three independent samples. ** P < 0.01; *** P < 0.001 relative to vehicle control or pervanadate stimulation. Experiments were performed in triplicate and repeated at least twice with similar results.

shedding was examined after electroporation of these cells with MUC1 cDNA or empty vector. Transfected wild-type MT1-MMP cells behaved similarly to HES cells with respect to constitutive and pervanadate-induced release of MUC1 (Figure 9A). MT1-MMP-deficient cells shed MUC1 constitutively; however, they failed to shed MUC1 after pervanadate stimulation (Figure 9B). To confirm that the lack of pervanadate-stimulated MUC1 ectodomain release in the MT1-MMP-deficient cells was solely due to the absence of MT1-MMP, we co-transfected the MT1-MMP-deficient fibroblasts with MUC1 and MT1-MMP cDNA. As shown in Figure 9(C), pervanadate-stimulated MUC1 shedding was restored in the co-transfectants.

MT1-MMP and TACE do not cleave MUC1 at the metabolic cleavage site *in vitro*

We compared the ability of MT1-MMP and TACE to cleave peptides representing the MUC1 metabolic cleavage site [37] in addition to putative cleavage sites of MUC1 within and outside the sea-urchin sperm protein, enterokinase and agrin module [38] (Table 2). Cleavage of each putative peptide substrate was determined after incubation with recombinant MT1-MMP or TACE by matrix-assisted laser-desorption ionization-time-of-flight MS. Neither MT1-MMP nor TACE cleaved the peptide spanning the intracellular metabolic site of cleavage. Nonetheless, TACE cleaved a synthetic peptide between an Ala-Ala peptide bond, corresponding to a potential cleavage site located 34-amino-acids upstream of the transmembrane domain of MUC1. In contrast, MT1-MMP did not cleave this peptide, suggesting a distinct site of action.

MUC1 and MT1-MMP co-localize in the receptive-phase human endometrium

Previous findings have indicated that MT1-MMP was most abundantly expressed in luminal and glandular epithelia of human uterus during the receptive phase of the menstrual cycle [36]. Moreover, our RT-PCR results also indicated that MT1-MMP was readily detectable in the receptive endometrium (Table 1). To establish whether MT1-MMP protein is appropriately expressed in the human uterus during receptivity to participate in MUC1 shedding, we examined expression of MT1-MMP and MUC1 in tissue sections from the receptive-phase human endometrium by immunofluorescence microscopy. MT1-MMP was expressed at the apical aspect of uterine epithelial cells with barely detectable

Figure 5 Pervanadate stimulates MUC1 shedding in TACE-deficient EC-2 cells

(A) Embryonic fibroblasts derived from wild-type (EC-4) mice were transiently transfected either with cDNA encoding full-length MUC1 (lanes 2–5) or with the vector control (lane 1) and treated with pervanadate or controls for the specificity of pervanadate-induced MUC1 release, VO_4 and H_2O_2 for 1 h at 37 °C. MUC1 expression in the culture supernatants (upper panel) and cell lysates (lower panel) was then examined by Western-blot analysis. Each experiment was performed in duplicate and repeated three times with similar results. (B) MUC1 shedding from TACE-deficient (EC-2) embryonic fibroblasts derived from $\text{tace}^{\Delta\text{Zn}/\Delta\text{Zn}}$ mice was determined as described for wild-type EC-4 cells in (A).

Figure 6 The synthetic metalloprotease inhibitor TAPI and the furin inhibitor decanoyl-RVKR inhibit pervanadate-stimulated MUC1 shedding in wild-type EC-4 and TACE-deficient EC-2 cells

(A, C) Embryonic fibroblasts derived from wild-type (EC-4) mice were transiently transfected either with cDNA encoding full-length MUC1 (lanes 2–5) or with the vector control (lane 1). After pretreatment with TAPI or decanoyl-RVKR for 1 h at 37 °C, wild-type EC-4 cells were treated with pervanadate or vehicle in the presence or absence of TAPI (A) or decanoyl-RVKR (C) for 1 h at 37 °C. MUC1 expression in the culture supernatants (upper panel) and cell lysates (lower panel) was then examined by Western-blot analysis. Each experiment was performed in duplicate and repeated three times with similar results. (B, D) MUC1 shedding from TACE-deficient EC-2 embryonic fibroblasts derived from $\text{tace}^{\Delta\text{Zn}/\Delta\text{Zn}}$ mice was determined as described for wild-type EC-4 cells above.

staining in the surrounding stromal cells (Figures 10A and 10G). Interestingly, this coincided with the predominant site of MUC1 localization (Figures 10B and 10E). The staining pattern for MUC1 and MT1-MMP was not observed with non-immune control antibodies (Figures 10D and 10H). Consequently, these results establish co-localization of MUC1 and MT1-MMP in epithelia of the receptive uterus (Figure 10C) and implicate MT1-MMP as a MUC1 sheddase *in vivo*.

DISCUSSION

We have demonstrated previously that ectodomain shedding of MUC1 is mediated through the activity of an ADAM, TACE/ADAM17. Nonetheless, it has been shown that a single protein

may be proteolytically processed by multiple proteases. In addition to cleavage mediated by TACE, $\text{TNF}\alpha$ also is a substrate for ADAM10 [39,40], MMP-7 [9] and MT4-MMP [10] in various cellular contexts. Furthermore, the nature of protease activation is influenced by the model system being studied and, for constitutive shedding, by the presence or absence of different stimuli. Before our studies, TACE-deficient cells have been used to examine alternative shedding mechanisms of TACE substrates, including transforming growth factor- α [41], TRANCE [11] and HER2 [24]. Constitutive and PMA-stimulated cleavage of these proteins was reduced to different degrees in the absence of TACE, although other types of stimuli, including pervanadate, were found to activate proteases distinct from TACE. These studies indicate that the model system used to study shedding activities constitutively

Figure 7 Pervanadate-stimulated MUC1 sheddase(s) in wild-type EC-4 and TACE-deficient EC-2 cells is sensitive to the endogenous metalloprotease inhibitors TIMP-2 and -3, but not TIMP-1

(A, C, E) Embryonic fibroblasts derived from wild-type (EC-4) mice were transiently transfected either with cDNA encoding full-length MUC1 (lanes 2–5) or with the vector control (lane 1). After pretreatment with TIMP-1, -2 or -3 for 1 h at 37 °C, wild-type EC-4 cells were treated with pervanadate or vehicle in the presence or absence of TIMP-1 (A), TIMP-2 (C) or TIMP-3 (E) for 1 h at 37 °C. MUC1 expression in the culture supernatants (upper panels) and cell lysates (lower panels) was then examined by Western-blot analysis. Each experiment was performed in duplicate and repeated three times with similar results. (B, D, F) MUC1 shedding from TACE-deficient EC-2 embryonic fibroblasts derived from *tace*^{ΔZn/ΔZn} mice was determined as described for wild-type EC-4 cells above.

Table 1 MT-MMP PCR profile in HES cells and in the receptive-phase endometrium

A qualitative measure was assigned to each PCR product based on the relative band intensity. +++, high expression; ++, intermediate expression; +, low expression; —, not expressed/not detected.

MT-MMP	Primer sequence (forward/reverse)	Reference for primer	Annealing temperature (°C)	PCR product size (bp)	Receptive-phase endometrium	HES cells
MT1-MMP	5'-GCTTGCAAGTAACAGGCAAA-3' 5'-AAATTCTCCGTGTCCATCCA-3'	[12]	58	589	+++	+
MT2-MMP	5'-TCGACGAAGAGACCAAGGAGT-3' 5'-CTTGAAGTTGTCAACGTCCT-3'	[12]	55	578	++	++
MT3-MMP	5'-ATGTGCTACAGTCTGCGGAAC-3' 5'-TATCCACATCACGTTTGCCA-3'	[12]	59	461	++	—
MT4-MMP	5'-TGC GTGCACTCATGTACTAC-3' 5'-GCCGCATGATGGAGTGTGCA-3'	[12]	55	334	++	++
MT5-MMP	5'-GGATCAGACAACGATCGAGT-3' 5'-CAGCTTGAAGTTGTGCTCT-3'	[12]	56	564	+	+
MT6-MMP	5'-TACGCTCTGAGCGGCAGC-3' 5'-CCCATAGAGTTGCTGCAG-3'	[52]	52	600	+	+/-

Figure 8 Overexpression of MT1-MMP enhances MUC1 release in HES uterine epithelial cells

(A) HES cells were transiently transfected either with cDNA encoding MT1-MMP (lanes 3 and 4) or with the vector control (lanes 1 and 2) and allowed to recover overnight. Cells were placed in fresh serum-free medium and treated with PV or vehicle for 1 h at 37 °C. MUC1 recovered from culture supernatants was then examined by Western-blot analysis. (B) Quantification of MUC1 shed into the medium was determined by densitometric analysis and is shown as a percentage of MUC1 released by cells treated with vehicle alone. Results are expressed as means $\pm$ S.D. for three independent samples of two separate experiments. * $P < 0.05$; *** $P < 0.001$ relative to vector vehicle control. Experiments were performed in triplicate and repeated at least twice with similar results.

or in response to different stimuli strongly influences the nature of protease activation. Thus we sought to determine whether different stimuli may activate MUC1 proteolytic activities in addition to TACE.

MUC1 is extremely resistant to externally added proteases. Therefore it is unlikely that cell-surface release of MUC1 is mediated by the actions of an external protease [42]. Moreover, the MUC1 metabolic cleavage complex is extremely stable [37,43,44]. Thus dissociation of the MUC1 heterodimer is unlikely, considering that mutant MUC1 transfectants devoid of the intracellular cleavage site would release little, if any, MUC1 if the mechanism of cell-surface release was simple dissociation [43]; however, a recent study indicates that the metabolic cleavage site is essential for MUC1 cell-surface release, at least in certain cellular contexts [45]. Nonetheless, this observation does not preclude an additional cleavage event from occurring at a later stage of processing, perhaps requiring the metabolic cleavage for appropriate recognition. Consistent with previous findings, studies conducted with several cell lines, including the HES uterine epithelial cell line, have demonstrated that MUC1 released from the cell surface is devoid of the cytoplasmic tail [20,44,46]. Furthermore, soluble MUC1 fragments have been found in bodily fluids, suggesting that proteolytic shedding may take place *in vivo* [47]. Additionally, release of MUC1 from HES cells is sensitive to metalloprotease inhibitors [20]. Collectively, these observations suggest that released MUC1 lacks the cytoplasmic tail and that release is catalysed by a protease(s).

Previous experiments utilizing hydroxamic acid-based metalloprotease inhibitors and the endogenous metalloprotease inhibitors, TIMP-1, -2 and -3, in addition to co-immunoprecipitation assays, suggested that TACE mediates aspects of MUC1 shedding in the human uterine epithelial cell line HES [20].

Figure 9 Pervanadate-stimulated shedding of MUC1 does not occur in MT1-MMP-deficient cells

(A) Embryonic fibroblasts derived from MT1-MMP wild-type mice were transiently transfected either with cDNA encoding full-length MUC1 (lanes 2 and 3) or with the vector control (lane 1) and treated with pervanadate or vehicle for 1 h. MUC1 in culture supernatants (upper panel) and cell lysates (lower panel) was then examined by Western-blot analysis. (B) MUC1 shedding from MT1-MMP null embryonic fibroblasts was determined as described for wild-type MT1-MMP cells in (A). (C) MT1-MMP null cells were co-transfected with cDNA encoding full-length MUC1 and MT1-MMP (lanes 2 and 3) or vector control (lane 1). Cells were treated with pervanadate or vehicle for 1 h. MUC1 recovered from the culture supernatants (upper panel) and in the cell lysates (lower panel) was assessed as described for wild-type MT1-MMP cells in (A).

Further evidence in support of TACE-mediated MUC1 ectodomain release is the complete absence of constitutive and PMA-stimulated MUC1 shedding in TACE-deficient cells, whereas both constitutive and stimulated release can be rescued by reconstitution of TACE expression [20]. In the present study, we have extended these studies by identifying a pervanadate-stimulated MUC1 sheddase(s) in HES uterine epithelial cells, wild-type EC-4 and TACE-deficient EC-2 cells, and wild-type and MT1-MMP-deficient mouse embryonic fibroblasts. Initially, utilizing HES cells, we found that MUC1 ectodomain release is much less sensitive to pervanadate when compared with PMA, suggesting that the MUC1 sheddase(s) is less active in the presence of pervanadate. Alternatively, a distinct protease(s), less

Table 2 Cleavage of peptides mimicking the MUC1 metabolic cleavage site and putative membrane-proximal cleavage sites by MT1-MMP and TACE

*, Predicted site of cleavage; –, no cleavage products were detected, i.e. only the intact peptide was detected; nt, not tested.

Substrate	Sequence	N-terminal produced by MT1-MMP	N-terminal peptide produced by TACE
Metabolic cleavage peptide	IKFRPG*SVVQL	–	–
Putative peptide 1	QYKTEA*ASRYNL	–	QYKTEA
Putative peptide 2	EGTIN*V*DVETQ	–	–
TACE-positive control peptide	Mca-PLAQA*V-Dpa-RSSSR-NH ₂	nt	PLAQA
MT1-MMP-positive control peptide	Mca-PLG*L-Dpa-AR-NH ₂	PLG	nt

active and/or less abundant than a PMA-sensitive sheddase(s), may mediate pervanadate-stimulated MUC1 ectodomain release. Pervanadate stimulation of MUC1 release is sensitive to the peptide hydroxamate metalloprotease inhibitors TAPI and GM6001, implicating a metalloprotease in pervanadate-stimulated MUC1 shedding. Interestingly, the furin inhibitor decanoyl-RVVR drastically attenuates stimulation of MUC1 shedding by pervanadate, but not by PMA [20], providing further support for an additional MUC1 sheddase. To characterize further the pervanadate-sensitive MUC1 sheddase activity and to determine the contribution of TACE, we examined MUC1 shedding in wild-type EC-4 and TACE-deficient EC-2 cells. Pervanadate stimulates MUC1 shedding in both cases, demonstrating that an activity distinct from TACE mediates pervanadate-induced MUC1 release. In addition, the pervanadate-responsive sheddase(s) was inhibited in both cell types by TIMP-2 and -3, but not by TIMP-1. Soluble MMPs are sensitive to both TIMP-1 and -2, excluding

Figure 10 Immunolocalization of MUC1 and MT1-MMP in tissue sections from the receptive-phase human endometrium

(A–C) Tissue sections were incubated with polyclonal anti-MT1-MMP and monoclonal anti-MUC1 (214D4) antibodies followed by incubation with FITC-conjugated goat anti-rabbit and Texas Red-conjugated donkey anti-mouse secondary antibodies. Nuclear (DAPI) staining is shown in the merged images in blue in each case (C, F, I). Glandular epithelial expressions of MT1-MMP (A), MUC1 (B) and the merged image (C) were visualized by confocal microscopy. (D–F) Tissue sections were incubated with non-immune rabbit IgG and the anti-MUC1 antibody followed by FITC-conjugated goat anti-rabbit and Texas Red-conjugated donkey anti-mouse secondary antibodies. Rabbit IgG (D), MUC1 (E) and the merged image (F) were processed as described in (A–C). (G–I) Tissue sections were incubated with the anti-MT1-MMP antibody and non-immune mouse IgG followed by FITC-conjugated goat anti-rabbit and Texas Red-conjugated donkey anti-mouse secondary antibodies. MT1-MMP (G), mouse IgG (H) and the merged image (I) were processed as described in (A–C).

MMPs as pervanadate-stimulated MUC1 sheddases [48]. Similarly, ADAM demonstrates variable sensitivities to these inhibitors [26,49,50]. Thus the MMPs and most of the currently known ADAM family members predicted to be catalytically active can be excluded as candidate pervanadate-stimulated MUC1 sheddases.

TIMP sensitivity of the pervanadate-stimulated MUC1 sheddase correlates with the TIMP inhibition profile of several MT-MMPs [25,34,35], in particular MT1- [34] and MT2-MMP [35]. Therefore MT1- and MT2-MMP were good candidates for pervanadate-stimulated MUC1 shedding. MT1- and MT2-MMP not only are inhibited by TIMP-2 and -3 [25,34], but also are expressed in HES cells and in the receptive-phase uterine epithelium. In fact, overexpression of MT1-MMP in HES cells leads to increased stimulation of MUC1 shedding by pervanadate. These observations suggest that in tissues where MUC1 and MT1-MMP are abundantly expressed, MUC1 may be a substrate for MT1-MMP. Unlike TACE-deficient cells that fail to shed MUC1 in the absence of PMA stimulation, constitutive MUC1 release by embryonic fibroblasts derived from MT1-MMP-deficient mice occurs normally. However, pervanadate-stimulated MUC1 release is abolished in MT1-MMP-deficient cells. Moreover, reconstitution of MT1-MMP expression in MT1-MMP-deficient cells restores pervanadate-stimulated MUC1 release, indicating that the defect in MUC1 shedding is not due to a genetic alteration other than the mutation of the MT1-MMP gene. Finally, *in vitro* cleavage studies indicate that a peptide spanning the membrane-proximal region of MUC1 is a substrate for TACE, but not MT1-MMP. Therefore it is possible that MUC1 is cleaved at different sites during constitutive and stimulated shedding events *in vivo*. In addition, physiological stimuli, triggering diverse signalling pathways, may activate alternate MUC1 sheddases. In contrast, neither TACE nor MT1-MMP cleaved a peptide spanning the known metabolic site of cleavage, indicating that it is unlikely that either of these metalloproteases participates in the intracellular metabolic cleavage event. Alternatively, these putative peptide substrates may not be cleaved well *in vitro*, implicating associations distal to the cleavage site as necessary in the regulation of MUC1 shedding.

Finally, previous findings have demonstrated that MUC1 is most abundantly expressed in the receptive-phase human endometrium [51]. Expression of MT1-MMP is also increased in uterine epithelia of the receptive-phase human endometrium [36]. In the present study, we demonstrated co-localization of MUC1 and MT1-MMP in human uterine epithelia from the receptive-phase endometrium. Therefore these results suggest that MT1-MMP is poised to participate in MUC1 ectodomain release *in vivo*, perhaps after stimulation by a physiologically relevant stimulus. Taken together, these findings are consistent with the role of MT1-MMP as a pervanadate-stimulated MUC1 sheddase. Future studies should provide additional insight into the physiological relevance of this proteolytic activity(ies). Moreover, a greater understanding of the mechanism regulating MUC1 cell-surface expression and release could lead to the development of approaches to promote selectively and/or prevent MUC1 shedding and, thus, modify MUC1-dependent processes in both normal and pathological contexts.

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